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Expression and stabilization of the *MCT-1* protein by DNA damaging agents

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The contribution of oncogene amplification and/or overexpression to T-cell lymphoid neoplasms has only of late been established with the implication of the *TCL1* and *MTCP1* genes in T-cell malignancies. Our laboratory has recently discovered a novel oncogene, *MCT-1*, amplified in a T-cell lymphoma and mapped to chromosome Xq22-24. *MCT-1* has been shown to decrease cell-doubling time, dramatically shortening the duration of G_1 transit time and/or G1-S transition, and transforms NIH3T3 fibroblasts. Constitutive expression of *MCT-1* results in a strong proliferative signal and is associated with deregulation of protein kinase-mediated G1/S phase checkpoints. In this study we analysed the level and subcellular localization of this novel cell cycle regulatory molecule as a function of cell cycle phase. In human lymphoid tumors expression of *MCT-1* is constant throughout the cell cycle and remains cytoplasmic. Cells overexpressing *MCT-1* have increased expression of cyclin D1 with dysregulation of the G_1-S checkpoint. Both cyclin D1 and *MCT-1* are involved in regulating passage of cells through the G1 phase of the cell cycle. Since prior work has shown that gamma irradiation induces cyclin D1 expression we investigated the induction of *MCT-1* to DNA damaging agents. We demonstrate that increases in *MCT-1* protein in irradiated human lymphoid cells do not occur at the mRNA level and do not require new protein synthesis. *Oncogene* (2001) **20,** 6777–6783.

Keywords: MCT-1 expression; stabilization; DNA damage

Introduction

Gene amplification is often associated with oncogenesis and drug resistance (Sandberg and Berger., 1994). The contribution of oncogene amplification and/or overexpression to T-cell lymphoid neoplasms has only of late been established (Ben-Yehuda *et al*., 1994). Recent reports have implicated the TCL1 and MTCP1 genes in leukemogenesis (Virgilio *et al*., 1994; Pekarsky *et al*., 1999; Sugimoto *et al*., 1999; Stern *et al*., 1993) Our laboratory has recently discovered a novel oncogene, *MCT-1*, amplified in a T-cell lymphoma and mapped to chromosome Xq22-24 (Prosniak *et al*., 1998). This region has been demonstrated to contain amplified DNA in a variety of lymphoid neoplasms using comparative genomic hybridization (CGH) (Monni *et al*., 1996; Werner *et al*., 1997). A recent report using both CGH and arbitrarily primed-PCR (AP–PCR) has identified several primary lymphomas with amplification of a common locus on chromosome X encompassing the region containing *MCT-1* (Scarpa *et al*., 1999). *MCT-1* has been shown to decrease cell doubling time, dramatically shortening the duration of G_1 transit time and/or G1-S transition, and transforms NIH3T3 fibroblasts (Prosniak *et al*., 1998; Dierov *et al*., 1999). While, there was no significant homology to *MCT-1* at the primary sequence level using the BLAST program (Altschul and Lipman, 1990) there were interesting alignments at the structural protein level (Prosniak *et al*., 1998). The amino terminal half revealed a sequence identity of 32% with a domain of cyclin H that appears to specify protein-protein complexes (Andersen *et al*., 1997). An intriguing finding is that all IL-2 independent T-cell leukemia/lymphoma lines examined to date exhibited elevated *MCT-1* protein levels in contrast to the IL-2 dependent T-cell lines which demonstrated low to absent *MCT-1* protein levels (Dierov *et al*., 1999). The G_1/S mitogen IL-2 appears to push T-lymphocytes through G_1/S transition without requiring high levels of *MCT-1* protein. This important association of growth factor independence with elevated *MCT-1* protein levels in lymphoid tumor lines led us to examine the effect of *MCT-1* overexpression on G_1 cyclin/cdk activity in *MCT-1* transformed cell lines. We observed an increase in the kinase activity of both cdk4 and cdk6 in exponentially growing *MCT-1* transformed cells compared to the parent control cells. An elevated level of cyclin D1 protein and increased G1 cyclin/cdk complex formation (Dierov *et al*., 1999) accompanied this increased kinase activity. These results demonstrated that constitutive expression of *MCT-1* results in a strong proliferative signal and is associated with

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deregulation of protein kinase-mediated G1/S phase checkpoints.

Because the expression of cell cycle regulatory molecules is often regulated during cell cycle progression, the expression of *MCT-1* may also be dependent on the phase of the cell cycle. In addition, the subcellular localization of some cell growth regulatory molecules has been shown to change depending on the phase of the cell cycle (Ye *et al*., 1998; Lam *et al*., 1997). In this study we analysed the level of *MCT-1* protein as a function of cell cycle phase. We also examined the subcellular localization of *MCT-1* in human lymphoid tumor cell lines using both asynchronous and synchronized cell populations.

We have previously demonstrated that cells overexpressing *MCT-1* have increased expression of cyclin D1 with dysregulation of the G_1-S checkpoint (Dierov *et al*., 1999). This G_1-S checkpoint dysregulation is associated with cells progressing through the cell cycle despite sustaining DNA damage (reviewed in Paulovich *et al*., 1997). An earlier report has demonstrated that irradiation induces cyclin D1 protein levels and that those cells overexpressing cyclin D1 have an increased percentage in S phase compared to the parent cell line (Epperly *et al*., 1995). The strong proliferative signal provided by MCT-1 raised the possibility that it too might override the G_1/S checkpoint as well. To investigate the potential role of *MCT-1* in this pathway we examined the induction of *MCT-1* after exposure to DNA damaging agents.

Results

MCT-1 *is predominantly localized to the cytoplasm in lymphoid cells and protein levels of* MCT-1 *are apparently stable throughout the cell cycle*

We have examined the subcellular localization of *MCT-1* in two human T-cell lymphoma cell lines using differential centrifugation followed by Western blotting with *MCT-1* antibody of the various subcellular fractions. As demonstrated in Figure 1, *MCT-1* was predominantly detected in the cytosolic fraction with slight staining in the heavy membrane fraction. We used the following antibodies; c-Myc, Bcl-2, NF-κB and Fas as controls for nuclear, heavy membrane, cytosolic and light membrane fractions, respectively.

Some cell growth regulatory proteins show different levels of expression as the cell proceeds through the cell cycle. We examined the level of *MCT-1* protein expression in lymphoid cell lines at different phases of the cell cycle. After arrest at the G1/S interphase using Aphidicolin, cells were released back into the cell cycle by washing and resuspension in growth medium. Levels of *MCT-1* protein expression and cell cycle profile were assessed by Western blot and flow cytometry of propidium iodide-stained cells, respectively. *MCT-1* protein levels in whole cell lysates essentially remained constant throughout cell cycle progression as demonstrated in Figure 2a. Similar results were obtained in two normal donor lymphocyte samples (data not shown).

Subcellular localization of MCT-1 *remains constant throughout the cell cycle*

The subcellular localization of some cell growth regulatory molecules have been shown to change depending on the phase of the cell cycle (Ye *et al*., 1998; Lam *et al*., 1997). After successful synchronization

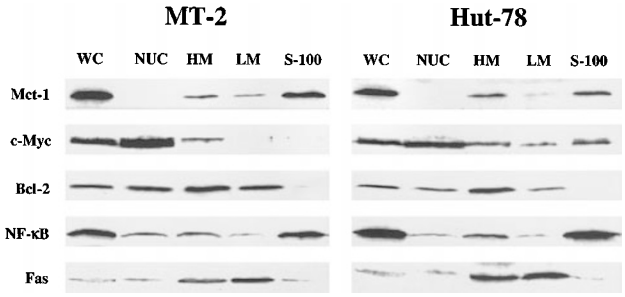

Figure 1 Subcellular localization of *MCT-1*. Subcellular fractionation was performed with isolation of the following fractions: WC-whole cell extract, Nuc-nuclear, HM-heavy membranes enriched in mitochondria, LM-light membranes enriched in endoplasmic reticulum and S-100- soluble fraction. Immunoblotting was carried out with the indicated primary antibodies. Observe the similar distribution of *MCT-1* in both cell lines. Controls included: c-Myc, Bcl-2, NF-κB and Fas as controls for nuclear, heavy membrane, cytosolic and light membrane fractions, respectively

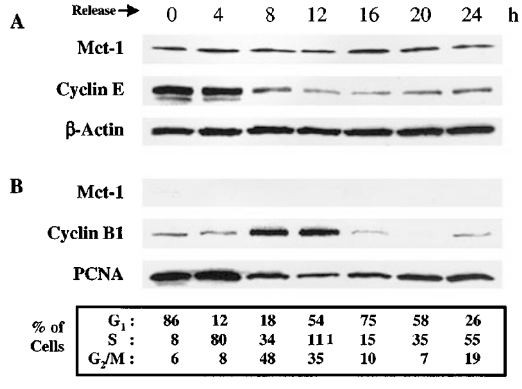

Figure 2 Levels of *MCT-1* protein and its subcellular localization remains constant throughout the cell cycle. (**a**) Jurkat cells are blocked at G_1/S phase boundary using 5 μg/ml Aphidicolin for 18 h. Following thorough washing cells were resuspended in complete RPMI and harvested at the indicated time points. Immunoblotting was performed with the indicated primary antibodies. Top: Observe the steady level of *Mct-1* protein as cells are released from aphidicolin arrest. Middle: Cyclin E levels upon release demonstrate the expected decrease from peak levels at the G_1-S boundary. Bottom: Equal loading of the gels is confirmed by the B-actin blot. (**b**) Analysis of nuclear fraction for *MCT-1* at different phases of the cell cycle. Jurkat cells were released from blockade and harvested at the indicated times. As shown, there is no detectable *Mct-1* protein in the nuclear fraction. Controls included Cyclin B that translocates to the nucleus from the cytoplasm in a cell cycle related manner with peak levels in the nucleus at G2/M. Additional controls included PCNA, its nuclear expression reaching a maximum in S phase as indicated

and release from G1/S block we analysed levels of *MCT-1* protein in both nuclear and cytoplasmic fractions. As shown in Figure 2b, the results are similar to the earlier analysis in asynchronous cells with no detectable levels of *MCT-1* in the nuclear fraction. Cyclin B was also examined since it has been previously shown to translocate to the nucleus from the cytoplasm in a cell cycle related manner with peak levels in the nucleus at G2/M (Figure 2b). Additional controls included PCNA, its nuclear expression reaching a maximum in S phase. Our results indicate that *MCT-1* protein does not translocate to the nucleus in cycling cells.

MCT-1 *displays a long half-life in human lymphoid cell lines*

The protein synthesis inhibitor cyclohexamide was added to two T-cell lines, MT-2 and Jurkat at a concentration of 5 μg/ml and remained present until harvest time. As demonstrated in Figure 3, the half-life of *MCT-1* in the MT-2 cell line is approximately 19 h. Similar results (not shown) were obtained in Jurkat cells. Controls included the p53 protein which is known to be stabilized in MT-2 cells and had a half-life of about 8 h as determined in our experiments consistent with published work (Reid *et al*., 1993; Gartenhaus and Wang, 1995).

MCT-1 *expression is rapidly induced in X-irradiated human lymphoid cells but not to other stressful stimuli such as heat shock*

In order to examine if *MCT-1* protein levels were effected by exposure to DNA damaging agents, we exposed exponentially growing normal donor PBL or relevant T-cell lines to one of the following; ionizing radiation (IR) using a ^{137}Cs source, adriamycin or taxol. Whole cell lysates (30 μg total protein) were prepared at the indicated time points for Western blot analysis with the indicated antibodies. We observe the rapid increase of *MCT-1* protein levels in normal donor lymphocytes as early as 2 h after exposure to these agents (Figure 4). This increase in *MCT-1* protein appeared not to require induction of p53 as demonstrated in the taxol treatment arm (Figure 4). A moderate dose response effect to gamma irradiation was observed with increasing induction of *MCT-1* protein levels as indicated in Figure 5a. Next, we examined two HTLV-1 transformed T-cell lines establishing that this DNA damage response is operative in both normal and transformed lymphocytes (Figure 5b). Finally, in order to address whether *MCT-1* protein induction was a general response to stress stimulus we examined the cellular levels of *MCT-1* after heat shock exposure. The Jurkat and EC155 T-cell lines were placed at 42°C for 45 min, protein lysates were prepared and examined by Western blot. As shown in Figure 6, there was induction of the control Hsp 27 in both cell lines but *MCT-1* protein levels remained constant. Thus, it appears that general stress stimuli such as heat shock do not induce *MCT-1*.

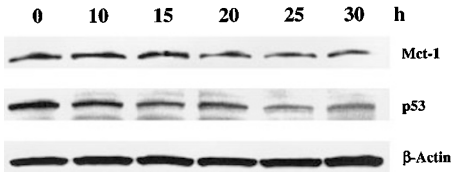

Figure 3 Half-life determination of *MCT-1*. The protein synthesis inhibitor cyclohexamide was added to the MT-2 T-cell line at a concentration of 5 μg/ml and remained present until harvest time. Protein quantification was carried out using laser densitometry (Molecular Dynamics), and half-life determination was calculated using standard methodology. The half-life of *MCT-1* in the MT-2 cell line is approximately 19 h. Controls included the p53 protein which is shown to be stabilized in MT-2 cells with a half-life of approximately 8 h. Virtually identical data were obtained in two independent experiments

The increase in MCT-1 *protein expression in irradiated human lymphoid cells does not occur at the mRNA level, and does not require new protein synthesis*

To investigate whether the induction of *MCT-1* protein expression in response to gamma irradiation is due to altered transcript levels we pretreated control and gamma irradiated normal lymphocytes with actinomycin D, a transcriptional inhibitor at 10 μg/ml. After 1 h pretreatment, cells were exposed to 10 Gy and analysed for levels of *MCT-1* protein. As shown in Figure 7, there was still significant induction of *MCT-1* protein at 2 h post-treatment. The control p21/Waf protein which increases in response to gamma irradiation primarily through a p53 dependent transcriptional mechanism was completely uninduced confirming the successful block of transcription. We also measured *MCT-1* mRNA levels in control and irradiated T-cells by Northern blot. There was no difference in mRNA levels post-irradiation (data not shown). These data exclude

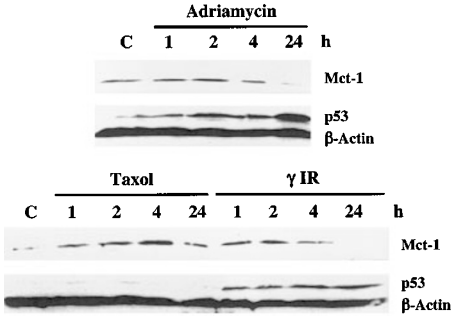

Figure 4 *MCT-1* expression is induced in response to DNA damaging agents and a microtubule-stabilizing agent. *MCT-1* protein levels in normal donor lymphocytes after exposure to adriamycin (10 μM), taxol (1 μM) and gamma irradiation (10 Gy). Treated cells were harvested at the indicated time points after exposure. Observe the significant *MCT-1* protein induction as early as 2 h post-treatment. Controls included the tumor suppressor p53 showing induction as expected after exposure to the DNA damaging agents but not after taxol, a microtubule-stabilizing agent. Experiments were independently performed at least four times with similar results

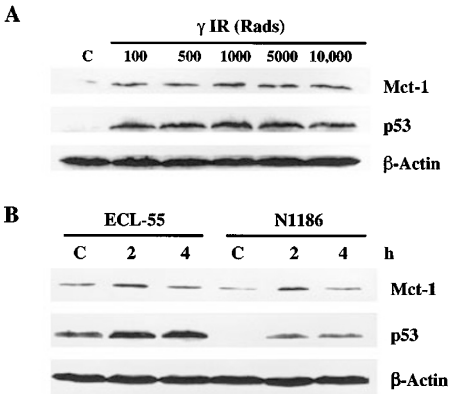

Figure 5 Normal human lymphocytes demonstrate a dose response induction of *MCT-1* to gamma irradiation and this response is also seen in transformed T-cell lines. (**a**) PBL exposed to the indicated dose of IR (1–100 Gy). Cell lysates from treated cells and untreated controls were obtained 2 h post-treatment and examined for MCT-1, p53 and B-actin protein levels. Observe the increase in MCT-1 protein correlating with increasing IR exposure. (**b**) Western blot Analysis of EC155 and N1186 after treatment with 1000 rads (10 Gy). Cell lysates from treated cells and untreated controls were obtained 2 h post-treatment and examined for MCT-1, p53 and B-actin protein levels. Experiments were independently performed at least three times with similar results

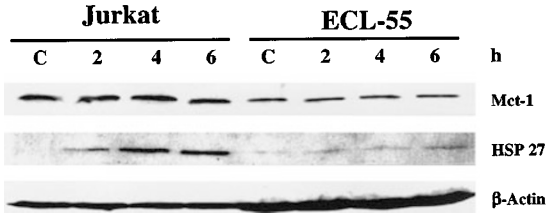

Figure 6 *MCT-1* expression is not induced by heat shock. In order to address whether *MCT-1* protein induction was a general response to stress stimulus we examined the cellular levels of *MCT-1* after heat shock exposure. The Jurkat and EC155 T-cell lines were placed at 42°C for 45 min, protein lysates were prepared and examined by Western blot. There was induction of the Hsp 27 in both cell lines though less so in the EC155 line. In both cell lines there was no induction of *MCT-1* protein levels observed after heat shock treatment. Therefore, it appears that general stress stimuli such as heat shock do not induce *MCT-1*

transcriptional/post-transcriptional regulation as a mechanism for the induction of *MCT-1* in response to gamma irradiation. We next investigated whether new protein synthesis is required for the up-regulation of *MCT-1* in response to gamma irradiation using the protein synthesis inhibitor, cyclohexamide. After pre-treatment with cyclohexamide (5 μg/ml) for 1 h, cells were irradiated and examined for induction of *MCT-1*. The data reveal an increase in the irradiated lymphocytes of *MCT-1* but not of p21/Waf (Figure 7). The inhibition of induction of p21/Waf attests to the successful translational arrest due to cyclohexamide and that increased expression of *MCT-1* protein must involve other mechanisms.

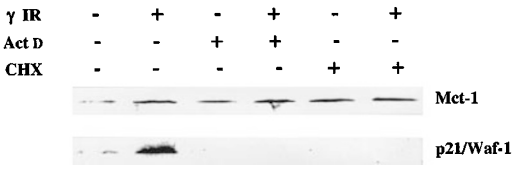

Figure 7 Altered *MCT-1* protein expression in irradiated cells does not occur at the mRNA level and does not require new protein synthesis. We pretreated control and gamma irradiated normal lymphocytes with actinomycin D, a transcriptional inhibitor at 10 μg/ml. After 1 h pretreatment, cells were exposed to 10 Gy and analysed for levels of *MCT-1* protein. At 2 h post-treatment there was significant induction of *MCT-1* protein. The p21/Waf protein was completely uninduced confirming the successful block of transcription. We next examined if the protein synthesis inhibitor, cyclohexamide could block up-regulation of *MCT-1* in response to gamma irradiation. After pre-treatment with cyclohexamide (5 μg/ml) for 1 h, cells were irradiated. The data reveal an increase in the irradiated lymphocytes of MCT-1 but not of p21/Waf. There was a modest increase after cyclohexamide alone in the level of *MCT-1* protein but a significantly greater increase after gamma irradiation was observed. The inhibition of induction of p21/Waf confirms that cyclohexamide was biochemically active in the treated cells and that increased expression of *MCT-1* protein must involve other mechanisms

Discussion

In previous work we screened a panel of T-cell leukemia/lymphoma lines using AP–PCR and identified an amplified DNA sequence in the Hut 78 lymphoid cell line (Prosniak *et al*., 1998). Expression analysis of a variety of normal human tissues revealed low level ubiquitous expression of *MCT-1* (Prosniak *et al*., 1998). The *MCT-1* cDNA sequence exhibited a high degree of evolutionary conservation across species (unpublished data). *MCT-1* is a novel gene which when overexpressed in cells shortens the time to progress through G_1/S phase, decreases cell doubling time and confers a transformed phenotype (Prosniak *et al*., 1998). Recent work has established increased levels of *MCT-1* protein in IL-2-independent T-cell leukemia/lymphoma cell lines relative to growth factor dependent T-cell lines (Dierov *et al*., 1999). In primary quiescent Chronic Lymphocytic Leukemia cells we found low to undetectable levels of *MCT-1* protein consistent with their arrest in G_0/G_1 (manuscript in preparation). The present study has examined the expression level of *MCT-1* throughout the cell cycle.

The expression of many cell cycle regulators including cyclins and cdk inhibitors vary according to the progression of the cell cycle. We consequently examined the cell cycle regulation of *MCT-1* protein levels at different phases of the cell cycle. As shown here, lymphoid cells arrested at G_1/S, then released and allowed to progress through the cell cycle display little variation of *MCT-1* protein during the cell cycle. The levels of cyclin E validate the successful synchronization as its levels decrease as expected upon release from G_1/S phase boundary blockade.

We examined the subcellular localization of *MCT-1* protein in two exponentially growing lymphoid cell lines using differential centrifugation to separate the various cellular fractions. We demonstrated that the major subcellular localization of *MCT-1* in asynchronous cell populations appears to reside in the cytosolic and light membrane fractions. It is highly reproducible in two distinct T-cell lines, HUT 78 and the HTLV-1 transformed MT-2.

To gain a better understanding of the possible mechanisms involved in *MCT-1* function, we also studied its intracellular localization as a function of the cell cycle. The subcellular localization of some cell cycle regulatory molecules has been shown to change depending upon the cell cycle stage (Petersen *et al*., 1999). We therefore examined the subcellular localization of *MCT-1* in T-cell lines that were synchronized at G_1/S phase boundary and released back into the cell cycle and analysed at various time points. Nuclear and cytoplasmic fractions were obtained (Poommipanit *et al*., 1999) for protein analysis. The cell cycle profile and *MCT-1* localization were monitored by flow cytometry of propidium iodide-stained cells and Western blotting. In two independent T-cell lines we observed no translocation of *MCT-1* to the nucleus throughout the cell cycle. These results are consistent with the lack of a nuclear localization signal in *MCT-1*. To verify the successful isolation of nuclei and cell cycle synchronization, we examined cyclin B since it has been previously shown to translocate to the nucleus from the cytoplasm in a cell cycle related manner with peak levels in the nucleus at G2/M. Analysis of PCNA further corroborated our findings with its nuclear expression peaking in S phase as previously reported.

In contrast to many cell cycle regulators, *MCT-1* levels remain constant during the cell cycle in proliferating cells, suggesting that the activity of the protein is restricted by another mechanism(s). It may be that there are post-translational modifications of *MCT-1* relative to the phase of the cell cycle. The *MCT-1* protein is predicted to have numerous putative phosphorylation sites including one tyrosine site, two PKC sites and a consensus cyclinB/cdc2 kinase substrate site (Prosniak *et al*., 1998; unpublished data). Phosphorylation has been shown to be involved in the regulation of many cellular processes, including protein-protein interactions, protein stability, kinase activity and protein localization. Additional studies will be required to determine the factors regulating *MCT-1* function during the cell cycle and to clarify its role in cell cycle progression.

Deregulation of the G_1-S checkpoint is associated with cells progressing through the cell cycle despite sustaining DNA damage. Both cyclin D1 and *MCT-1* are involved in regulating passage of cells through the G1 phase of the cell cycle. A previous report has shown that gamma irradiation induces cyclin D1 expression (Epperly *et al*., 1995) In order to examine if *MCT-1* protein levels were also modulated by exposure to gamma irradiation, we exposed exponentially growing normal donor PBL to ionizing radiation (IR) using a ^{137}Cs source and consistantly observed a 3–5-fold increase in *MCT-1* protein. The dose of 10 Gy was chosen as this has reproducibly demonstrated induction of p53 in our T-cell lines as well as in normal PBL. It should be noted that the induction of *MCT-1* was not as dramatic as induction of p53 or its downstream target p21. We extended these studies to include another DNA damaging agent, adriamycin. Similar results were obtained with induction of *MCT-1* protein as early as 2 h after exposure. In order to determine whether this response was restricted to DNA damaging agents, we employed Taxol, a microtubule-stabilizing agent. Interestingly, there was a comparable amount of *MCT-1* induction. The p53 tumor suppressor gene does not appear to be required for *MCT-1* induction as illustrated in the taxol treatment arm where we observed no induction of p53. Furthermore, we demonstrated that MCT-1 induction was not simply a universal sensor of cellular stress as evidenced by our heat shock experiments. Since taxol works at clinically relevant doses primarily through its tubulin-binding properties (Chaung *et al*., 1994) and downstream serine phosphorylation of proteins including Bcl-2 (Blagosklonny and Fojo, 1999), our findings suggest potential avenues of research. For example, the *MCT-1* protein has at least one potential serine phosphorylation site and will be examined for phosphorylation modifications both before and after treatment. Our experiments performed with actinomycin and cyclohexamide indicate that increases in *MCT-1* protein in irradiated human lymphoid cells does not occur at the mRNA level and does not require new protein synthesis.

The cellular response to genotoxic stress is complex and it is unclear at present the role that *MCT-1* plays in this response. A recently described RNA-binding domain, designated PUA domain (Aravind and Koonin, 1999) has been identified in the *MCT-1* protein (Gastenhaus and Shi, manuscript in preparation). This novel domain has been associated with the translation machinery and has also been detected in a family of eukaryotic proteins that also contain a domain homologous to the translation initiation factor eIF1 (Sheikh *et al*., 1999). It has been suggested that modulation of translation initiation may be an important adaptive response to genotoxic and other cellular stress (Sheikh *et al*., 1999). Further work is required in order to examine the role of *MCT-1* in the cellular response to genotoxic stress and the specific function of the PUA domain.

The similar pattern of *MCT-1* protein induction in response to two distinct stimuli i.e., ionizing radiation and a microtubule-stabilizing agent suggests alternative signaling pathways converging downstream. Our aggregate data support the hypothesis that induction of *MCT-1* at least in response to irradiation is through increased stabilization by post-translational mechanism(s) unknown at present.

Materials and methods

T-cell lymphoid cell lines and peripheral blood lymphocytes

T-cell lymphoid tumors included both IL-2 dependent; EC155 (Advanced Biotechnologies, Columbia, MD, USA) NII85 and N1186 (Berneman *et al*., 1992) and IL-2 independent; Hut 78, MT-2 and Jurkat (Advanced Biotechnologies, Columbia, MD, USA). All cell lines were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Life Technologies, Inc) and IL-2 dependent T-cell lines were also supplemented with 40 μ/ml of recombinant IL-2 (Life Technologies, Inc). Normal donor peripheral blood lymphocytes (PBL) were centrifuged over Ficoll-Hypaque (Pharmacia, Pleasant Hill, CA, USA) isolating mononuclear cells. After PHA stimulation the lymphocytes were cultured in a similar manner as the IL-2 dependent T-cell lines.

Cell cycle synchronization

Fully confluent Jurkat cells were blocked at the G1/S interphase using 5 μg/ml aphidicolin for 18 h. Following a thorough washing of the cells they were subsequently resuspended in growth medium and harvested at the indicated time points for analysis of *MCT-1* protein levels as a function of the cell cycle. In some instances we employed a double aphidicolin block. Briefly, logarithmically growing cells were blocked in S phase overnight with 1 μg/ml aphidicolin (Sigma), washed twice in complete RPMI 1640 and then released into fresh, prewarmed complete RPMI 1640. Following the completion of S phase, cells were again blocked overnight at the G_1/S border with 5 μg/ml of aphidicolin. Cells were washed three times and released into fresh, prewarmed complete media for time-course analysis. Cells from each time point were washed twice with ice cold PBS. An aliquot of these cells was fixed and stored in 70% ethanol at -20°C for cell cycle analysis, a second aliquot was used to make whole cell lysates, while the remaining cells were used to isolate nuclei. The viability of cells at the end of the time course was $\geqslant$98% as determined by trypan blue exclusion. Fluorescence data were collected with the Coulter Epics XL-MCL flow cytometer and the percentage of cells within the G_1, S, and G_2-M phases of the cell cycle was determined by analysis with the software program MultiCycle (Phoenix).

Preparation of cellular fractions

Cells were lysed in hypotonic buffer (42.5 mM KCl, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) by passaging them four times through a 30-gauge needle. Isotonicity was reestablished by adding an equal volume of hypertonic buffer (242.5 mM KCl, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.4). Nuclei and unlysed cells were pelleted twice at 200 *g* for 10 min. The supernatant was centrifuged at 10 000 *g* for 10 min to collect the heavy membrane pellet. That supernatant was centrifuged at 100 000 *g* for 60 min, and the final supernatant was collected as the soluble fraction, and the pellet was collected as the light membrane fraction. The heavy membrane pellet was washed twice in H medium (0.25 mM mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4, and 0.1% fatty acid-free bovine serum albumin). Both heavy and light membrane pellets were lysed in RIPA buffer and, together with the soluble fraction, were stored at -70° for immunoblotting.

Nuclear isolation Cells for nuclear isolation were washed twice in hypotonic buffer (10 mM HEPES, pH 7.5, 5 mM KCl, 2 mM $MgCl_2$), resuspended in hypotonic buffer plus 1 mM PMSF, 0.5 mM DTT, and protease inhibitor cocktail (1 : 20, Sigma) and swelled on ice for 15 min. Nuclei were released by subjecting cells to 5–15 strokes with a Dounce homogenizer (pestle B), washed twice with hypotonic buffer, lysed in sample buffer, boiled for 5 min and clarified by centrifugation at 16 000 *g* for 15 min. The integrity and purity of nuclei for each preparation was verified by trypan blue and was $\geqslant$99%.

Immunoblotting Cells lysates were prepared for immunoblotting as follows: cells were washed twice in PBS, lysed in sample buffer (62.5 mM Tris, pH 6.8, 2% SDS) and boiled for 5 min. Sample viscosity was reduced by passage through a 20 ga needle and lysates were clarified by centrifugation at 16 000 *g* for 15 min. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA, USA) and 20 μg of protein were separated on 12.5% gels by SDS–PAGE and transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) + 5% nonfat dried milk for 1 h at room temperature and then incubated with primary antibody diluted in block buffer for 1 h at room temperature. Blots were then washed three times (10 min each) with TBST and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse IgG or donkey anti-rabbit IgG, diluted 1 : 5000 in TBST (Amersham Pharmacia Biotech) or an anti-goat IgG diluted 1 : 7000 in block buffer (Santa Cruz, Santa Cruz, CA, USA)) for 1 h. Blots were again washed three times in TBST and specific proteins were detected by ECL (Amersham Pharmacia Biotech). Primary antibodies included a rabbit polyclonal anti-*Mct-1* (Research Genetics), a mouse monoclonal anti-Bcl-2 (Santa Cruz), a rabbit polyclonal anti-Fas (Santa Cruz), a mouse monoclonal anti-β-actin (Sigma, St. Louis, MO, USA), a rabbit polyclonal anti-c-Myc (Santa Cruz), a goat polyclonal anti-NF-κB (Santa Cruz), a rabbit polyclonal anti-cyclin E (Santa Cruz), a mouse monoclonal anti- PCNA (Santa Cruz), a mouse monoclonal anti-p53 (Santa Cruz), a mouse monoclonal anti-Hsp27 (Santa Cruz) and a mouse monoclonal anti-cyclin B1 (Santa Cruz). Equal concentrations of protein from the various subcellular fractions were loaded unto gels with quantitation determined by using laser densitometry.

DNA damage and heat shock stress response In order to examine if *MCT-1* protein levels were effected by exposure to DNA damaging agents, we exposed exponentially growing normal donor PBL or relevant T-cell lines to ionizing radiation (IR) using a ^{137}Cs source (1–100 Gy) and harvested cells at the indicated time points for Western blot analysis, protein levels were quantitated by laser densitometry (Molecular Dynamics) beginning at 2 h after IR exposure. We also examined the effect of Adriamycin (10 μM) and taxol (1 μM) on cellular levels of *MCT-1*. The p53 protein was analysed to confirm adequate delivery of a DNA damage dose. In order to determine if heat shock exposure would induce *MCT-1*, the Jurkat and EC155 T-cell lines were placed at 42°C for 45 min and cell lysates were examined by Western blot.

References

AltShul SF and Lipman DJ. (1990). *Proc. Natl. Acad. Sci. USA,* **87,** 5509–5513.

Anderson G, Busso D, Poterszman A, Hwang JR, Wustz JM, Ripp R, Thiessy JM, Egly JM and Mosas D. (1997). *EMBO J.,* **16,** 958–967.

Aravind L and Koonin EV. (1999). *J. Mol. Evol.,* **48,** 291–302.

Ben-Yehuda D, Houldsworth J, Parsa NZ and Chaganti RSK. (1994). *Br. J. Haematol.,* **86,** 792–796.

Berneman ZN, Gartenhaus RB, Reitz Jr MS, Blattner WA, Manns A, Hanchard B, Ikehara O, Gallo RC and Klotman ME. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 3005–3009.

Blagosklonny MV and Fojo T. (1999). *Int. J. Cancer,* **83,** 151–156.

Chuang LT, Lotzova E, Heath J, Cook KR, Munkarah A, Morris M and Wharton TJ. (1994). *Cancer Research,* **54,** 1286–1291.

Dierov J, Prosniak M, Gallia G and Gartenhaus RB. (1999). *J. Cell. Biochem.,* **74,** 544–550.

Epperly M, Berry L, Halloran A and Greenberger JS. (1995). *Radiat Res.,* **143,** 245–254.

Gartenhaus RB and Wang P. (1995). *Leukemia,* **9,** 2082–2086.

Lam MHC, Olsen SL, Rankin WA, Ho PWM, Martin TJ, Gillespie MT and Moseley JM. (1997). *J. Cell. Physiol.,* **173,** 433–446.

Monni O, Joensuu H, Franssila K and Knuutila S. (1996). *Blood,* **87,** 5269–5278.

Paulovich AG, Toczyski DP and Hartwell LH. (1997). *Cell,* **88,** 315–321.

Pekarsky Y, Hallas C, Isobe M, Russo G and Croce CM. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 2949–2951.

Petersen BO, Lukas J, Sorensen CS, Bartek J and Helin K. (1999). *EMBO J.,* **18,** 396–410.

Poommipanit PB, Chen B and Oltvai ZN. (1999). *J. Biol. Chem.,* **274,** 1033–1039.

Prosniak M, Dierov J, Okami K, Tilton B, Jameson B, Sawaya BE and Gartenhaus RB. (1998). *Cancer Res.,* **58,** 4233–2337.

Reid RL, Lindholm PF, Mireskandari A, Dittmer J and Brady JN. (1993). *Oncogene,* **8,** 3029–3033.

Sandberg AA and Berger CS. (1994). *J. Urol.,* **151,** 545–556.

Scarpa A, Taruscio D, Scardoni M, Iosi F, Paradisi S, Ennas MG, Rigaud G, Moore PS and Menestrina F. (1999). *Genes Chromosomes Cancer,* **26,** 203–209.

Sheikh MS, Fernandez-Salas E, Yu M, Hussain A, Dinman JD, Peltz SW, Huang Y, Fornace Jr AJ. (1999). *J. Biol. Chem.,* **274,** 16487–16493.

Stern MH, Soulier J, Rosenzwajg M, Nakahara K, Canki-Klain N, Aurias A, Sigaux F and Kirsch IR. (1993). *Oncogene,* **8,** 2475–2483.

Sugimoto J, Hatakeyama T, Narducci MG, Russo G and Isobe M. (1999). *Cancer Res.,* **59,** 2313–2317.

Virgilio L, Narducci MG, Isobe M, Billips LG, Cooper MD, Croce CM and Russo G. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 12530–12534.

Werner CA, Dohner H, Joos S, Trumper LH, Baudis M, Barth TF, Ott G, Moller P, Lichter P and Bentz M. (1997). *Am. J. Pathol.,* **15,** 335–342.

Ye Y, Vasavada S, Kuzmin I, Stackhouse T, Zbar B and Williams BRG. (1998). *Int. J. Cancer,* **78,** 62–69.